

# Immune Responses to AAV in Clinical Trials

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**Abstract:** Recent findings in a clinical trial in which an adeno-associated virus (AAV) vector expressing coagulation factor IX (F.IX) was introduced into the liver of hemophilia B subjects highlighted a new issue previously not identified in animal studies. Upon AAV gene transfer to liver, two subjects enrolled in this trial developed transient elevation of liver enzymes, likely as a consequence of immune rejection of transduced hepatocytes mediated by AAV capsid-specific CD8<sup>+</sup> T cells. Studies in healthy donors showed that humans carry a population of antigen-specific memory CD8<sup>+</sup> T cells probably arising from wild-type AAV infections. The hypothesis formulated here is that these cells expanded upon re-exposure to capsid, i.e. upon AAV-2 hepatic gene transfer, and cleared AAV epitope-bearing transduced hepatocytes. Other hypotheses have been formulated which include specific receptor-binding properties of AAV-2 capsid, presence of capsid-expressing DNA in AAV vector preparations, and expression of alternative reading frames from the transgene. Absence of a valid animal model has prevented an in-depth mechanistic study of the phenomenon. Several possible solutions to the problem are discussed, including the administration of a short-term anti-T cell immunosuppression regimen concomitant with gene transfer. While more studies will be necessary to further define mechanisms and risks associated with capsid-specific immune responses in humans, monitoring of these responses in clinical trials will be essential to achieving the goal of long-term therapeutic gene transfer in humans.

**Keywords:** Adeno-associated virus, AAV, immune response, gene therapy, clinical trial, CD8 T cells, capsid, liver.

## INTRODUCTION

Over the past ten years, considerable efforts have been invested in establishing safe and effective gene therapy approaches for the treatment of a variety of diseases. Indeed, proof of concept of the great potential of gene transfer has been demonstrated in several preclinical studies in animal models of diseases [Biffi *et al.*, 2006; Brown *et al.*, 2006; De Meyer *et al.*, 2006; Follenzi *et al.*, 2004; Jiang *et al.*, 2006b; Mount *et al.*, 2002; Ponder *et al.*, 2002]. However, translation of results from bench to bedside has been successful in only a few cases [Aiuti *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2002], while clinical research highlighted several major obstacles that need to be overcome. These include: 1) gene silencing [ESGT, 2006]; 2) insertional mutagenesis [Hacein-Bey-Abina *et al.*, 2003]; 3) phenotoxicity, i.e. problems arising from either overexpression or ectopic expression of the donated gene; 4) immunotoxicity, i.e. harmful immune responses to either the vector or to the transgene product [Manno *et al.*, 2006; Mingozzi *et al.*, 2007b]; 5) risks of horizontal transmission of the donated DNA; and 6) risks of vertical transmission, i.e. inadvertent germline transmission of the donated gene sequences [High, 2003].

Adeno-associated virus (AAV) vectors have been widely adopted as gene delivery vehicles because of their ability to transduce a wide variety of tissues, mediating long-term expression of the donated gene after a single *in vivo* administration [Arruda *et al.*, 2005; Herzog *et al.*, 1997; Jiang *et al.*,

2006b; Mount *et al.*, 2002]. Wild-type AAV is not associated with any disease pathology in humans, and is also naturally replication-defective, requiring a helper virus such as adenovirus to replicate [Muzyczka *et al.*, 2001]. AAV vectors are one of the simplest gene therapy vectors, containing only the transgene expression cassette flanked by two non-coding viral inverted terminal repeats (ITRs) enclosed in a capsid composed of three structural proteins, VP1, 2, and 3 [Samulski *et al.*, 1982]. The simplicity of AAV vectors, and their low efficiency in transducing professional antigen presenting cells (e.g. macrophages or dendritic cells) [Jooss *et al.*, 1998; Vandendriessche *et al.*, 2007; Zaiss *et al.*, 2002] perhaps contribute to their generally low immunogenicity. Moreover, transferred genomes tend to persist inside the cells mainly in an episomal, non-integrated form, reducing the chances of insertional mutagenesis [Duan *et al.*, 1998; Nakai *et al.*, 2001].

A large number of studies in experimental animals have established the potential of AAV vectors as a therapeutic tool [Acland *et al.*, 2001; Adriaansen *et al.*, 2005; Flotte, 2005; Ghosh *et al.*, 2007; Goyenvalle *et al.*, 2004; Herzog *et al.*, 1997; Jiang *et al.*, 2006; Jiang *et al.*, 2006b; Mas *et al.*, 2006; Mount *et al.*, 2002; Song *et al.*, 1998]. However, translation of these results into clinical studies revealed some of the limits of animal models in fully predicting outcomes in humans, a finding by no means unique to gene transfer therapeutics [Suntharalingam *et al.*, 2006].

The use of AAV vectors in the clinical setting revealed two main issues not anticipated by animal studies. The first of these was detection of vector sequences in the semen of vector recipients [Manno *et al.*, 2006], raising the specter of germline transmission of the donated gene [Boyce, 2001;

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<http://www4.od.nih.gov/oba/RAC/meeting.html>, 2002]; the establishment of a rabbit model which accurately reflected the human findings and predicted that spermatocytes would not be transduced and that vector sequences would be cleared from the semen over time [Arruda *et al.*, 2003; Schuettrumpf *et al.*, 2006] addressed this issue, completing the bench-to-bedside-and-back cycle and correctly predicting the outcome of human studies. The second issue is related to immunotoxicity; findings in a clinical trial in which an AAV vector was introduced into the liver of hemophilia B subjects [Manno *et al.*, 2006; Mingozzi *et al.*, 2007b] indicate that immune response directed to the AAV capsid represents one of the last major roadblocks to the development of a successful therapeutic platform based on AAV-mediated gene transfer.

In this article we shall review the data available on host immune responses to the AAV capsid in humans, the current hypothesis formulated to explain differences between humans and experimental animals, and strategies proposed to avoid capsid recognition by the human immune system.

### IMMUNE RESPONSES TO AAV CAPSID IN GENE TRANSFER FOR HEMOPHILIA

The lack of circulating functional coagulation factor VIII (F.VIII) or factor IX (F.IX) results in the X-linked inherited disease hemophilia A or B, respectively [Kazazian *et al.*, 2001; Pollak *et al.*, 2001]. Hemophilia is characterized by defective coagulation, resulting in increased risk of bleeding into joint spaces, with consequent arthropathy, or in bleeding in other internal sites, exposing patients to life-threatening hemorrhagic episodes. Although a conventional, protein replacement therapy is available to manage hemophilia, several limitations of this treatment prompted the study of a gene replacement approach to treat the disease [High, 2001]. Furthermore, hemophilia represents an ideal disease model for gene therapy as: a) clinical endpoints are well defined and b) these are easily measured; c) circulating levels between 5 and 100% of normal result in significant amelioration of the disease phenotype and are not associated with side effects; d) functional clotting factors can be produced by a variety of tissues, including liver, muscle, and fibroblasts [Arruda *et al.*, 2001; High, 2001; Palmer *et al.*, 1989; Snyder *et al.*, 1999]. However, one severe complication of both conventional, protein replacement therapy, and (potentially) gene therapy is the development of inhibitory antibodies (termed inhibitors) to the coagulation factor, which severely complicate clinical management of the disease [DiMichele, 2007; Hay *et al.*, 2006].

### AAV-Mediated Gene Transfer to Skeletal Muscle

Based on pre-clinical studies in mice [Herzog *et al.*, 1997] and hemophilic dogs [Herzog *et al.*, 1999] demonstrating the safety and efficacy of direct intramuscular injection of an AAV vector expressing F.IX, the first clinical trial of parenterally administered AAV vectors was carried out [Kay *et al.*, 2000; Manno *et al.*, 2006]. Hemophilia B subjects carrying missense mutations in the F.IX gene were enrolled in three dose cohorts receiving  $2 \times 10^{11}$  to  $1.8 \times 10^{12}$  vector genomes (vg)/kg. After direct intramuscular administration of an AAV-2 vector encoding human F.IX under the control

of a CMV promoter, long-term (>3 years) transgene expression was achieved, based on immunofluorescence staining of biopsied injected muscle [Jiang *et al.*, 2006c]. However, at the doses tested, circulating F.IX levels did not reliably rise above 1%.

No acute and long-term toxicity was observed following vector administration, documented by normal clinical laboratory testing, including absence of inhibitory and non-inhibitory antibodies to the secreted transgene product.

Neutralizing antibodies (NAb) to the AAV-2 capsid were identified in 7 of the 8 subjects enrolled, and in 5 subjects the titer was  $\geq 1:100$ , clearly indicating exposure to AAVs prior to study initiation. In all subjects the NAb titer increased by 2-3 logs after vector administration and remained 1-2 logs higher than baseline for several years, confirming that AAV capsid antigen did not escape recognition by the immune system. Previous exposure to AAV-2 did not seem to affect muscle transduction and did not seem to be associated with cytotoxic T cell responses to AAV capsid, documented by the absence of muscle enzyme elevation and absence of lymphocytic infiltrates in repeated muscle biopsies analyzed over several years. However, activation of AAV capsid-specific T cells upon gene transfer cannot be completely ruled out as no specific immunological assays (e.g. ELISPOT) were performed in this study.

Importantly, all subjects had been exposed to HIV, HBV, and/or HCV as a consequence of prior infusion with infected plasma-derived products but this did not alter the outcome of gene transfer.

### AAV-Mediated Gene Transfer to Liver

Compared to other target tissues, liver presents several advantages as a target for gene transfer. First, it is the natural site of biosynthesis of clotting factors, so the necessary post-translational modifications take place accurately and efficiently; second, being a highly vascularized organ, therapeutic proteins newly produced in the liver can easily and efficiently gain access to the systemic circulation, conferring a dose advantage compared to muscle as documented by animal studies; third, expression of antigens in transduced hepatocytes is associated with induction of tolerance rather than immunity. Hepatic administration of an AAV vector expressing human F.IX results in sustained expression without neutralizing antibody formation, even in animals not previously tolerant to the transgene product [Mingozzi *et al.*, 2003; Mount *et al.*, 2002]. In contrast to results in muscle-directed AAV gene transfer, liver-directed AAV gene transfer results in antigen-specific immunological unresponsiveness to the transgene product in several strains of mice. Tolerance can be transferred to naïve recipients through adoptive T cell transfer after hepatic gene transfer with AAV and once established is not broken by immunological challenge with F.IX in adjuvant. Furthermore, studies in DO11.10 mice transgenic for the ovalbumin (OVA) T cell receptor, showed that hepatic gene transfer of an AAV-OVA vector induces antigen-specific  $CD4^+CD25^+$  regulatory T cells [Cao *et al.*, 2007; Dobrzynski *et al.*, 2004]. Similar results have been confirmed by several labs using a variety of transgenes, delivery vectors, and animal models [Nathwani *et al.*, 2007; Wang *et al.*, 2005]. Finally, beyond hemophilia, the estab-

ishment of a gene transfer technology platform for hepatic gene transfer would allow the treatment of a wide range of genetic disorders and infectious diseases.

The pre-clinical work supporting AAV-mediated gene transfer to liver for hemophilia B is compelling [Mount *et al.*, 2002]. Long-term expression (>5 years) at therapeutic levels (6-8% of normal levels) in hemophilic dogs was achieved after infusing a dose of  $1 \times 10^{12}$  vg/kg of AAV-canine F.IX into the portal vein without production of neutralizing antibodies to the transgene product (Lothrop and High, unpublished data).

With the expectation that immune responses to the transgene product would be avoided in a liver approach, the first dose escalation study of hepatic artery delivery of an AAV-2 vector expressing human F.IX under the control of a liver-specific promoter was initiated [Manno *et al.*, 2006]. Subjects affected by severe hemophilia B (pre-treatment F.IX levels <1% of normal) were enrolled into three dose cohorts, receiving  $8 \times 10^{10}$  to  $2 \times 10^{12}$  vg/kg. Vector infusion proceeded uneventfully for all subjects.

None of the subjects enrolled in the first two dose cohorts showed evidence of vector-related toxicity or efficacy defined as levels of human F.IX expression >1% of normal. The 5<sup>th</sup> subject, subject E, was the first enrolled at what was expected to be a therapeutic dose ( $2 \times 10^{12}$  vg/kg) based on the canine studies. This individual did indeed show therapeutic levels of F.IX expression initially, in the range of 10-12%, sufficient to convert his disease phenotype from severe to mild. However, beginning 4 weeks after vector infusion, F.IX levels began to fall, and gradually returned to baseline (<1%) at 10 weeks after vector infusion. Concurrently, liver enzymes, which had been normal for the first few weeks after vector infusion, began to rise, then slowly returned to normal without medical intervention. No inhibitory or non-inhibitory antibodies to the F.IX transgene product were measured in this subject. Possible toxic or infectious causes of the increase in liver enzymes were sought and excluded. At the request of regulatory agencies, the next subject studied, subject G, was infused at a 5-fold lower dose than E and also experienced an asymptomatic, self-limited liver enzyme elevation, with similar kinetics.

ELISPOT analysis of T cell responses in peripheral blood mononuclear cells (PBMC) isolated from subject G demonstrated IFN- $\gamma$  production in response to AAV capsid peptides, but not to F.IX peptides [Manno *et al.*, 2006]. Identification of AAV capsid peptide epitopes to which subjects E and G reacted, allowed the synthesis of MHC class I pentamers for the direct quantitation of capsid-specific CD8<sup>+</sup> T cell populations in peripheral blood [Mingozzi *et al.*, 2007b]. This population was shown to expand then contract after vector infusion, with a time course that closely matched the rise and fall of serum transaminases [Mingozzi *et al.*, 2007b]; moreover, even long (>2 years) after vector exposure, although capsid-specific CD8<sup>+</sup> T cells are no longer detectable in peripheral blood by ELISPOT, *in vitro* expansion of lymphocytes with the relevant epitope in the presence of IL-2 and IL-7 produces a robust expansion of a population of capsid-specific CD8<sup>+</sup> T cells, indicating the presence of a pool of memory T cells, which through homeostatic proliferation are maintained long after gene transfer.

Importantly, AAV capsid-specific CD8<sup>+</sup> T cells generated by *in vitro* expansion against AAV peptide epitopes or AAV empty capsids (whole capsid protein) have the ability to lyse HLA-matched peptide-loaded or AAV vector-transduced human hepatocytes [Basner-Tschakarjan *et al.*, 2007] in an *in vitro* CTL assay, indicating a) these cells are antigen specific and fully-functional, and b) human hepatocytes can process and present AAV-derived epitopes at their surface.

Interestingly, presence of neutralizing antibodies (NAb) to the AAV-2 capsid prior to vector infusion had a profound effect on liver transduction. Subjects E and F both received the highest dose of vector, however only subject E, who had low pretreatment NAb, achieved appreciable levels of transduction, while F, who had a titer of 1:17 did not. Subject F did not experience an increase in liver enzymes. Similarly, among subject C, D, and G, who all received the same vector dose, only subject G, with the lowest pretreatment NAb to AAV-2, had a spike in liver enzymes [Manno *et al.*, 2006]. These data indicate that even low levels of NAb can prevent transduction when the AAV vector is introduced through the systemic circulation, and that the presence of antibodies to AAV is not predictive of T cell responses against AAV capsid.

#### THE CAPSID-SPECIFIC MEMORY CD8<sup>+</sup> T CELL REACTIVATION HYPOTHESIS

Our hypothesis to account for the decline in F.IX levels, the rise and fall of liver enzymes, and the expansion and contraction of a capsid-specific CD8<sup>+</sup> T cell population, is a CD8<sup>+</sup> T cell response to capsid that recognizes and destroys the transduced hepatocytes. One question raised by this work is why human subjects manifest T cell responses to AAV capsid after vector infusion, while animal models do not [Mingozzi *et al.*, 2007b]. Prior exposure to AAV capsid probably underlies the difference in response. A high percentage of humans, the only natural hosts for wild-type AAV-2 infection, are infected by AAV-2 in childhood (*vide infra*). Because AAV is naturally replication defective, this initial infection invariably takes place together with a helper virus infection such as adenovirus. Although AAV-2 on its own may not induce the inflammatory reactions needed for stimulation of a maximal adaptive immune response, in combination with the helper virus, which causes activation of the innate immune system, it is likely that CD8<sup>+</sup> T cells directed to the antigens of both the helper virus and of AAV are formed. Upon controlling the infection, the frequency of AAV-specific CD8<sup>+</sup> T cells would be expected to decline, leaving behind a small pool of memory T cells, which through homeostatic proliferation are maintained throughout the lifetime of an individual. On re-exposure to capsid, these memory CD8<sup>+</sup> T cells are activated and eliminate the AAV capsid-harboring cells (the transduced hepatocytes). Because memory T cells are more readily triggered than naïve lymphocytes, human subjects, undergoing re-exposure, have an outcome different from experimental animals, undergoing what amounts to a primary infection with AAV.

Additional factors contributing to the difference in outcome between humans and other species are discussed in a later section of this article.

## PREVALENCE OF B AND T CELL IMMUNITY TO CAPSID IN THE GENERAL POPULATION

Studies on the prevalence of neutralizing antibodies (NAb) in cystic fibrosis and healthy donor populations [Chirmule *et al.*, 1999; Erles *et al.*, 1999; Halbert *et al.*, 2006; Mingozi *et al.*, 2007b] show a relatively low prevalence of NAb to AAV-2 early in life (10-20% of subjects between 0 and 10 years of age are positive), which then increases rapidly with age to around 30% of adult subjects with NAb titer to AAV-2 >1:20. However, it is also important to note that even if only a relatively small portion of the population is positive for NAb, a much larger proportion of adults have detectable antibodies (neutralizing and non-neutralizing) to AAV, indicating widespread exposure to the virus early in life.

Prevalence of T cell responses in healthy subjects has been evaluated in two separate studies (summarized in Table 1). In the first study, Chirmule and colleagues [Chirmule *et al.*, 1999] measured <sup>3</sup>H-thymidine incorporation (proliferation) and IFN- $\gamma$  production by PBMC in response to AAV capsid; in this study 6% of subjects (3/57) had detectable responses. In the second study, performed by our lab [Mingozi *et al.*, 2007b], a peptide library derived from the AAV capsid was used to stimulate lymphocytes, and T cell responses were measured by IFN- $\gamma$  ELISPOT on PBMC isolated from healthy donors; similarly to the previous study, ~4% of subjects (2/46) showed positive responses to the AAV-2 capsid. When human splenocytes isolated from spleens removed for non-malignant diseases were used as starting material, ~7% (2/28) of subjects had positive responses to AAV-2 peptides. However, upon *in vitro* expansion with either peptide epitopes derived from the AAV capsid protein or whole capsid, ~28% (2/7) of PBMC and 60% (9/15) of spleen samples became positive for T cell responses to AAV-2, indicating that: a) AAV-specific T cells fail to circulate in peripheral blood but rather reside in lymphoid organs; b) in general frequency of these T cells is low and therefore not detectable by ELISPOT, the most sensitive assay available for this purpose. These findings have clear implications for the feasibility of pre-screening of subjects undergoing AAV-mediated gene therapy.

Additional evidence that humans carry a pool of expandable AAV-specific memory CD8<sup>+</sup> T cells (*vide supra*) comes from immunophenotyping studies performed in normal donors, which allowed the identification of a population of resting central memory CD8<sup>+</sup> T cells able to produce IFN- $\gamma$  in response to AAV-derived peptide epitopes [Mingozi *et al.*, 2007b].

## EXPERIENCE FROM OTHER AAV GENE TRANSFER CLINICAL TRIALS

Of the over 500 human subjects that received AAV vectors (mainly AAV serotype 2) [Warrington *et al.*, 2006] to date, the vector was introduced in the systemic circulation only in subjects enrolled in the hemophilia liver trial [Manno *et al.*, 2006]; moreover, this is the only investigation in which a detailed study of T cell responses to AAV capsid has been described [Mingozi *et al.*, 2007b].

Other groups have now adopted protocols similar to the hemophilia B muscle trial [Kay *et al.*, 2000; Manno *et al.*, 2003] for studies of AAV gene transfer for lipoprotein lipase deficiency,  $\alpha_1$ -antitrypsin deficiency [Brantly *et al.*, 2006], and muscular dystrophies. Most of these results have not yet been published. Preliminary results from a clinical trial in which an AAV-1 vector was used to deliver an expression cassette encoding lipoprotein lipase to skeletal muscle of subjects missing the enzyme, indicate the possibility that T cell responses to AAV capsid may be activated upon gene transfer [Mingozi *et al.*, 2007c]. In another study, subjects affected by  $\alpha_1$ -antitrypsin deficiency received intramuscularly an AAV-2 vector encoding the missing enzyme; in this study no responses to the AAV-2 capsid were detected [Brantly *et al.*, 2006]. However, data are preliminary and in the two studies T cell responses were measured with assays with different sensitivity (ELISPOT vs. T cell proliferation assay).

Interestingly, in a recently reported phase I clinical study for Parkinson's disease [Kaplitt *et al.*, 2007], after injection of small amounts of an AAV-2 vector encoding glutamic acid decarboxylase into the subthalamic nucleus, no change in NAb titer to AAV-2 was observed, compared to pretreatment levels. This suggests that gene delivery with small

**Table 1. AAV-Specific T Cell Responses in Normal Human Subjects**

Source of Cells	Assay	Positive/total	Reference
PBMC	Lymphocyte proliferation measured by <sup>3</sup> H-thymidine incorporation. ELISA on cell supernatant for IFN- $\gamma$ production in response to AAV capsid	3/57 (~6%)	[Chirmule <i>et al.</i> ]
PBMC	IFN- $\gamma$ ELISpot on unexpanded cells	2/46 (4%)	[Mingozi <i>et al.</i> ]
Splenocytes from patients undergoing splenectomy for non-malignant diseases	IFN- $\gamma$ ELISpot on unexpanded cells	2/28 (7%)	[Mingozi <i>et al.</i> ]
PBMC	IFN- $\gamma$ ELISpot on cells expanded with peptides or whole capsid	2/7 (28%)	[Mingozi <i>et al.</i> ]
Splenocytes	IFN- $\gamma$ ELISpot on cells expanded with peptides or whole capsid	9/15 (60%)	[Mingozi <i>et al.</i> ]

amounts of AAV vectors in immunoprivileged sites (like brain or eye) would escape immune B and T cell responses to capsid; this is discussed at greater length in the review by Lowenstein *et al.* elsewhere in this volume [Lowenstein *et al.*, *Current Gene Therapy*, 2007]. Similarly, gene transfer to tissue targets with low MHC class I expression may limit the exposure of vector antigen to immune recognition.

### LACK OF ANIMAL MODELS TO STUDY IMMUNE RESPONSES TO CAPSID

Independent findings recently published by three groups [Li *et al.*, 2007; Li *et al.*, 2007; Wang *et al.*, 2007] indicate that animals fail to develop T cell responses to AAV capsid. Mice immunized against the AAV capsid by either an intramuscular injection of an adenovirus expressing AAV capsid or by repeated injection of dendritic cells pulsed with capsid do indeed develop AAV-specific cytotoxic T lymphocytes, however, these primed CD8<sup>+</sup> T cells fail to clear *in vivo* AAV-transduced hepatocytes. This difference between experimental animals and humans may be explained by the greater responsiveness of human T cells to T cell receptor stimulation due to the loss of sialic acid-recognizing Ig-superfamily lectins on human T cells [Nguyen *et al.*, 2006], better homing of primed human T cells to liver [Lang *et al.*, 2006], inefficient presentation of capsid antigen in murine liver, or, alternatively, murine models in which populations of AAV-primed effector CD8<sup>+</sup> T cells, rather than memory CD8<sup>+</sup> T cells, predominate. These data confirm observations in hundreds of animal studies in which long-term expression of the transgene was documented after AAV-mediated gene transfer. The lack of an animal model to study T cell responses to capsid represents a major limitation to understanding the mechanism[s] of this phenomenon. Development of murine models in which the liver can be repopulated with human hepatocytes [Azuma *et al.*, 2007] may afford a solution.

### ALTERNATE HYPOTHESES

Alternate hypotheses proposed to explain the findings in the hemophilia B AAV liver trial have included packaging into the vector of residual plasmid expressing capsid (which is one of the plasmids used in AAV vector production, [Matsushita *et al.*, 1998]), resulting in continuous expression of this viral protein in the transduced hepatocytes, and destruction on that basis, or translation of alternate open reading frames within the F.IX expression cassette, leading to production of immunogenic foreign proteins [http://www4.od.nih.gov/oba/RAC/meeting.html, 2007]. Although encapsidation of prokaryotic sequences during AAV vector production has been documented [Chadeuf *et al.*, 2005], studies performed on clinical lots of vector utilized in AAV-infused subjects who developed transaminitis revealed only very low levels (<1%) of DNA other than the AAV expression cassette in viral preparations. Additionally, in these experiments no evidence of capsid expression has been found *in vitro* and *in vivo* after transducing cell lines at high MOIs or injecting mice with AAV vectors at high doses [http://www4.od.nih.gov/oba/RAC/meeting.html, 2007]. Furthermore, small and large animal experiments performed with AAV vectors identical to those used for clinical application never evi-

denced these problems, arguing against this as the explanation.

Another hypothesis proposes that a specific motif (heparin binding domain) within the dominant protein of the AAV-2 capsid favors receptor-mediated uptake of vector by human dendritic cells, with activation of capsid-specific CD8<sup>+</sup> T cells occurring on that basis [Vandenberghe *et al.*, 2006]. This hypothesis predicts that alternate serotypes of AAV, like AAV-8, lacking this motif, will avoid the capsid-specific CD8<sup>+</sup> T cell responses seen in the earlier study [Manno *et al.*, 2006]. Although the capsid sequences are fairly heavily conserved (60-98%), it is indeed possible that subtle differences in kinetics of vector uncoating, patterns of intracellular trafficking, or other factors effecting antigen presentation, may allow an alternate serotype to escape destruction by the immune response. However, experiments performed with human PBMC, either from AAV-infused subjects or normal donors, expanded *in vitro* with AAV-derived peptide epitopes or whole capsids from different serotypes, indicate that capsid-primed CD8<sup>+</sup> T cells cross-react when they encounter alternate serotypes [Mingozzi *et al.*, 2007b]. Moreover, since dendritic cells have the ability to micropinocytose and present antigen [Norbury, 2006] and, in addition to that, since presentation by dendritic cells is not essential for activation of memory CD8<sup>+</sup> T cells, differential uptake of alternate serotypes by dendritic cells does not seem likely to be a determinant of capsid T cell responses in previously exposed human subjects.

### POSSIBLE SOLUTIONS TO THE PROBLEM

Different strategies could be effective in escaping, or limiting, T cell responses to AAV capsid in humans. The development of an animal model (*vide supra*) would allow further analysis of some of these approaches.

#### Alternate Serotypes

Multiple serotypes of AAV have been isolated, either as contaminants of adenoviral isolates, or from non-human primate tissues by PCR, using primers designed within known conserved sequences from previously identified AAVs [Gao *et al.*, 2002]. Conservation of capsid sequences among serotypes ranges from 63-100%; differences among capsids result in utilization of different cell surface receptors on the host cell, giving rise to distinct tissue tropisms for each serotype. Similarly, directed evolution of AAV capsid [Maheshri *et al.*, 2006] and generation of serotypes that are hybrids between known serotypes [Hauck *et al.*, 2006] may result in enhanced gene transfer vectors. However, results in humans (*vide supra*) indicate that AAV capsid-primed CD8<sup>+</sup> T cells are likely to cross-react with alternate serotypes [Mingozzi *et al.*, 2007b]; this finding, together with the high degree of conservation of AAV capsid protein sequence, and the complexity of the human HLA antigen recognition system, make these approaches less likely to succeed in avoiding immune responses.

#### Modulate T Cell Responses to Capsid

While the idea of inducing tolerance to the AAV capsid in individuals undergoing AAV-mediated gene therapy is intriguing, the feasibility of this approach is questionable in a clinical setting. A short course of immunosuppression (IS),

given around the time of gene transfer, has been proposed. Pre-clinical studies in a non-human primate (NHP) model [Mingozzi *et al.*, 2007a], however, highlighted the importance of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) in establishing and maintaining tolerance to transgene product, confirming previous data developed in mice [Cao *et al.*, 2007; Dobrzynski *et al.*, 2004; Mingozzi *et al.*, 2003]. In these studies, the use of an anti-CD25 monoclonal antibody at the time of AAV-2 hepatic gene transfer, as part of an anti-T cell regimen based on mycophenolate mofetil (MMF) and sirolimus, likely interfered with Tregs homeostasis, resulting in neutralizing antibodies to the transgene product. Exclusion of the anti-CD25 antibody from the regimen resulted in long-term expression of transgene without neutralizing antibody formation, confirming the safety of AAV-2 hepatic gene transfer in the context of an IS regimen based on MMF and sirolimus. Discussion of additional strategies for reducing immune responses to the transgene product after gene transfer are contained elsewhere in this volume in the review by Miao [Miao, Current Gene Therapy 2007].

### Alter Half-Life of Capsid Antigen within Transduced Cells

Complete understanding of the fate of AAV capsid from entry into the cell to the trafficking to the nucleus will open new avenues to prevent antigen presentation in the context of MHC class I molecules on the surface of transduced target cells, possibly avoiding activation of the immune system. Protein-capsid engineering aimed at decreasing the half-life of the antigen, and therefore the window of time during which peptide epitopes will be present on the surface of transduced target cells and recognizable by the immune system can serve this scope; alternatively, introduction of motifs that prevent trafficking of capsid to proteosomes may be effective in preventing antigen presentation of MHC class I molecules.

### Select Naïve Subjects

Selecting subjects that never encountered the AAV capsid antigen may be challenging both because available assays are not sensitive enough and also because data collected in humans indicate that exposure to wild-type AAV occurs at age 5 or earlier. Furthermore, the only starting material available for screening of human subjects are PBMC, while data available on frequency of capsid-specific T cell responses [Mingozzi *et al.*, 2007b] suggest that a higher frequency of these responses can be found by testing human splenocytes, suggesting that capsid specific memory CD8<sup>+</sup> T cells fail to circulate in peripheral blood (*vide supra*).

### Reduce Antigenic Load

Data from the hemophilia B liver clinical trial in the two subjects who developed transient elevation of liver enzymes suggest a direct relationship between the dose of vector administered and the peak levels of liver enzymes [Manno *et al.*, 2006]. Thus, diminishing the amount of antigen (i.e. vector capsid) introduced into the human organism may help to evade immune responses, as will introduction of vector into closed, immunoprivileged sites [Acland *et al.*, 2001; Kaplitt

*et al.*, 2007]. Therapeutic levels of transgene expression may be achieved at lower vector doses using more efficient expression cassettes [Liu *et al.*, 2004], AAV serotypes with higher tropism for the target tissue [Gao *et al.*, 2002; Mingozzi *et al.*, 2002], or self-complementary AAVs [McCarty *et al.*, 2001; Nathwani *et al.*, 2007; Nathwani *et al.*, 2006]. Moreover, since, on average, AAV vector preparations contain a ratio of full:empty capsids of 1:3 to 1:100 (only full capsids contain the expression cassette), efforts from a manufacturing standpoint should be aimed at producing empty capsid-free clinical-grade vector to significantly reduce the antigen load [Qu *et al.*, 2007].

### CONCLUSIONS

Clinical experience with AAV vectors is still at an early stage, and translation of preclinical results into humans highlighted a previously unknown problem related to immunogenicity of AAV capsid. Data developed in humans suggest that the problem observed in a clinical trial for AAV-2-mediated hepatic gene transfer for hemophilia may be seen also in other trials, with other AAV serotypes, and with other target tissues. On the other hand, experience to date indicates that injection of small doses of AAV vector into immunoprivileged sites [Kaplitt *et al.*, 2007] may be tolerated uneventfully, thus leading to long-term expression of transgene.

A difficulty in interpreting clinical data to date is the lack of easily quantifiable endpoints; exceptions to this include plasma proteins such as F.IX and  $\alpha_1$ -antitrypsin. It is possible that reports suggesting efficacy at early time points, followed by loss of efficacy [Moss *et al.*, 2007] are actually reflecting undiagnosed immune-mediated elimination of transduced cells. Again, for some target organs such as liver, or skeletal or cardiac muscle, immune-mediated destruction of transduced cells is easily appreciated by measuring relevant serum enzyme levels, while for other cell types such as those lining the respiratory tract, this is more difficult to diagnose.

Future studies will need to address fundamental questions such as: a) is there a reliable way to predict which subjects will react to AAV capsid; b) are some serotypes, delivery methods, and target tissues more or less immunogenic than others; c) what are the biomarkers that can predict immune rejection of transduced cells; finally, and most importantly d) what is the fate of the AAV capsid inside the transduced cell and how long does the capsid persist in an immunologically detectable form.

Capsid-specific ELISPOT screening for all subjects undergoing AAV-mediated gene transfer is warranted [<http://www4.od.nih.gov/oba/RAC/meeting.html>, 2007] and, if performed, data produced in the only relevant model available to date, humans, will contribute to the full understanding of the mechanisms and risks associated with T cell responses to AAV capsid.

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## ABBREVIATIONS

AAV	= Adeno-Associated Virus
F.IX	= Factor IX
ELISPOT	= Enzyme-Linked Immunosorbent Spot
OVA	= Ovalbumin
CTL	= Cytotoxic T Lymphocyte
NAb	= Neutralizing Antibody
PBMC	= Peripheral Blood Mononuclear Cell
MHC	= Major Histocompatibility Complex
MOI	= Multiplicity Of Infection
NHP	= Non-Human Primate
MMF	= Mycophenolate Mofetil

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